ELECTROPORATION DEVICE

TECHNICAL FIELD

The above invention relates to an electro - poration device.

BACKGROUND ART

As it is known, recent biological, microbiological and pharmacological applications involve introducing molecules into cells, which is done by introducing the molecules through the cell membranes.

The molecules may be inorganic substances (e.g. drugs) or organic molecules (DNA molecules for example are known to be introduced in cells).

In order to introduce the molecules, the so-called electroporation methods have recently been devised, which are based on the application of electric pulses to the cells in order to produce an electric field that permeabilizes the cell structure enabling the substances to penetrate the cell membrane.

For instance PCT patent application **WO01/07583** describes an Electro - poration device wherein an electrical voltage is applied to a substrate comprising cells and a current is flowing through the substrate. The above patent application also proposes to continuously detect the ratio of the current through the substrate to the voltage across the substrate as an

indication of the obtained degree of electroporation of the substrate.

Finally, application **WO01/07583** proposes to adjust the magnitude of the applied voltage in accordance with changes in the above ratio to achieve a controlled degree of electroporation in the substrate.

However, patent application **WO01/07583** does not teach how to use the information related to the above ratio in order to have useful information for controlling the voltage; in other words, **WO01/07583** merely discloses the possibility of controlling the voltage based on the detection of the above ratio but does not provide any real indication for creating a working system wherein the control of the voltage is successfully performed.

DISCLOSURE OF INVENTION

Scope of the present invention is to provide a working electroporation device wherein the control of the voltage applied to the substrate is obtained by means of the continuos monitoring of the ratio of the current flowing through the substrate and the voltage applied to the substrate.

According to another embodiment of the present invention, the control of the voltage is obtained by simply monitoring the current.

The above scope is obtained by the present

invention as it relates to an electro poration device as described in claim 1 or 13.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention shall be described in accordance with the attached drawings which show a non-limiting embodiment of the invention wherein:

- figure 1 shows, in a simplified manner, an electroporation device according to the present invention;
- figure 2 is a flow chart of the operations performed by the electro poration device according to a first embodiment of the invention;
- figure 3 illustrates a signal ratio of current to voltage based on which the control of the electroporating pulse S(t) is performed; and
- figure 4 is a flow chart of the operations performed by the electro poration device according to a second embodiment of the invention.

BEST MODE FOR CARRYING OUT THE INVENTION

In figure 1, number 1 indicates an electro - poration device realised according to the present invention.

In particular, device 1 comprises of a signal generator 3 for producing a stimulating signal S(t) that

is amplified by a power amplifier 5 and applied to electrodes 6, 7 through a power switch 10. The electrodes 6,7 are coupled with a substrate 12 containing living cells C; in particular, the substrate 12 may comprise a portion (for instance a tissue) of a living body (plant, animal or man) or may comprise a substrate separate from the living body and contained in a recipient (for instance a culture of animal, plant, bacterial or fungal cells in vitro).

The application of the electroporating signal S(t) to the electrodes 6,7 causes the creation of an electric field E(t) in the substrate 12; such a field E(t) promotes, realises or enhances the permeabilization of the membranes of the cells C rendering possible the introduction of molecules (organic/inorganic) through the membranes of the cells C.

Device 1 also comprises a measuring signal generator 15 coupled with a measurement block 16 co-operating with electrodes 6, 7 for the determination of the instantaneous values of the current through the substrate 12, the voltage actually applied to the substrate 12, and the ratio **GT** of the current through to voltage applied to the substrate 12 (i.e. $\mathbf{GT=i_e/v_p}$).

Signal generator 3, measuring signal generator 15 and measurement block 16 are connected, through a common BUS 20, with a central processing unit 23 coupled with

interface circuits (not shown), for the communication with peripheral devices, such as a video display 25, keyboard 26 and printer 27.

Under the control of the central processing unit 23 the signal generator 3 generates a signal S(t) comprising one or a series of pulses which amplitude may be regulated as described in the following. Moreover, the central processing unit 23 receives the information related to the Voltage V_p of the stimulating signal S(t) applied to the electrodes 6,7 and the current i_e that flows between the electrodes 6,7 through the substrate 12. More particularly, central processing unit 23 detects the instantaneous value of the ratio GT of the current i_e flowing through the substrate 12 and the voltage **Vp** applied to the substrate 12, i.e.: $GT = i_e/v_p$. and determines the instantaneous value of the signal S(t).

The knowledge of the above ratio GT is used for controlling stimulating signal **S(t)** as described in the flow chart of figure 2.

Studies and experiments of the applicant have revealed that the curve C_{GT} representing the value of ratio GT in successive instants after the application of the stimulating signal S(t) has a particular waveform that is shown, as non limiting example, in figure 3.

In the Cartesian presentation of figure 3, Y-axis

represents increasing values or ratio GT and X-axis represents successive instants after the start of the application of the stimulating signal S(t); the stimulating signal S(t) being applied from time t = 0 on.

Curve $\mathbf{C_{GT}}$ (if permeabilization process is in progress) comprises a first portion $\mathbf{C_{GT}}$**-I** decreasing from time t = 0 to a time t = **Tm** wherein an initial minimum is reached, a second portion $\mathbf{C_{GT}}$**-II** increasing from the time **Tm** wherein the minimum is reached, and a third portion $\mathbf{C_{GT}}$**-III** increasing with a very low rate or being substantially flat.

Curve $\mathbf{C_{GT}}$ (if permeabilization is not achieved and permeabilization process in not in progress) comprises a first portion $\mathbf{C_{GT}}$**-I** decreasing from time t = 0 (the second portion $\mathbf{C_{GT}}$**-II** increasing from the time **Tm** is absent) and a third portion $\mathbf{C_{GT}}$**-III** decreasing with a very low rate or being substantially flat.

In particular, block 100 of figure 2 commands the generation of the stimulating signal S(t) and the application of such a signal S(t) to the substrate 12 through electrodes 6 and 7.

Block 100 is followed by a block 110 that introduces a delay **Td** so that the stimulating signal S(t) is applied for at least a predetermined period of time **Td**; such a period of time **Td** having a value so

that ratio **GT** has time to reach and overcome its initial minimum value **Tm**, in particular ratio **GT** at the end of delay **Td** is placed on the second portion C_{GT}**-II** of curve C_{GT}.

Block 110 is followed by block 120 that determines the instantaneous gradient **dG** (i.e. instantaneous slope) of the ratio GT after the minimum has been reached, i.e. calculates the derivative of ratio GT, **dG = d(GT)/d(t)**, at the beginning of the second portion C_{GT}**-II**, or more practically the difference **deltaG=deltaGT/deltaT** at **Tm**.

Block 120 is followed by block 130 that compares the calculated instantaneous variation **dG** of the ratio **GT** with a reference value dG_{ref1}, for instance dG_{ref1} **= 1**.

In particular, if the calculated instantaneous variation **dG** of the ratio **GT** is greater than the reference value dG_{ref1} (for instance d**G > 1)** block 130 is followed by block 140. If the applied signal **S(t)** is of too small amplitude to initiate the process of permeabilization the first minimum is not reached within the predetermined time Tm and the gradient at Td is lower than a predetermined dG_{ref2}, for instance dG_{ref2}**=0**, the **dG** at **Td** is negative (**dG<0**) and block 130 is followed by block 180, whilst if the calculated instantaneous variation **dG** of the ratio **GT** is smaller than the reference value dG_{ref1} and at the same time larger than dG_{ref2}(for instance 0 < d**G < 1)** block 130 is

followed by block 150.

Block 140 performs an urgent correction to the stimulating signal S(t) in order to avoid lesions, damages or irreversible alterations in substrate 12; to that regard block 140 is followed by a block 145 that decreases the amplitude (i.e. the voltage) of the stimulating signal S(t) in order to prevent deterioration in the cells C. Block 145 is then followed by block 110.

Block 150 calculates the average variation **ΔG** of ratio **GT** (i.e. the slope calculated over a period of time) in a time interval that is successive to the instant **Tm** wherein the minimum has been reached and that has a pre-determined time width, for instance **ΔG = ΔGT/(T1-Tm)** wherein **T1 > Tm**.

Block 150 is followed by a block 160 that compares the calculated average variation **ΔG** of ratio **GT** with a reference interval of **ΔG** values, for instance a reference interval having limits **0** and ΔG_{obb}, wherein ΔG_{obb} is an expected value of the average variation **ΔG** above which the corresponding pulses will lead to a too intense permeabilization of the cells and to subsequent damages to the cells.

In particular block 160 performs the following functions:

- if the calculated average variation **ΔG** of ratio

GT falls within the reference interval (for instance $0 < \Delta G < \Delta G_{obb}$) then block 160 is followed by a block 170;

- if the calculated average variation **ΔG** of ratio **GT** falls outside the reference interval and it is smaller than both the limits delimiting the interval (for instance $\Delta G < 0 < \Delta G_{obb}$) then block 160 is followed by a block 180; and
- if the calculated average variation **ΔG** of ratio **GT** falls outside the reference interval and it is greater than both the limits delimiting the interval (for instance $\Delta G > \Delta G_{obb} > 0$) then block 160 is followed by block 140.

Block 180 increases the voltage of the stimulating signal in order to increase the value of the electric field E(t) applied to the substrate 12; block 180 is then followed by block 110.

Block 170 increases the voltage of the stimulating signal to an objective voltage V_{opt} in order to increase the value of the electric field E(t) applied to the substrate 12 so that the value of **ΔG** tends to the expected value ΔG_{obb}.

When the objective voltage V_{opt} has been reached block 170 is followed by a block 190 that maintains the stimulating signal at the objective voltage V_{opt} for a predetermined time that is sufficient for achieving a

complete electroporation of cells C.

Finally, block 190 may be followed by a block 195 that stops the application of pulses of the stimulating signal S(t) or by a block 200 that, if needed, continues for an extra period of time the application of the stimulating signal of the same or of another value (for example, lower), depending on the molecule to be introduced.

Figure 4 shows another preferred embodiment of the present invention. The operations that have not been modified have been indicated by blocks having the same reference numbers, and different operations are indicated with new block and numbers.

More particularly, block 120 is followed by block 125 that detects the minimum of the ratio GT. This operation may be realized according know techniques for instance by comparing the instantaneous gradient dG calculated with previously recorded gradients.

Block 125 is followed by block 126 that determines the time Tm at which the minimum detected by block 125 occurs.

Block 126 is followed by block 127 that compares the detected time Tm with threshold values Ttmin and Ttmax.

More particularly, if the detected Tm occurs before Ttmin (Tm < Ttmin) then block 126 is followed by block

140.

If the detected Tm occurs after Ttmax (Tm > Ttmax), then block 126 is followed by block 180.

If Tm occurs between Ttmin and Ttmax, then block 126 is followed by block 150. Accordingly block 130 is eliminated.

In actual use, electrodes 6, 7 are applied to the substrate 12 (shown schematically in Figure 1) containing live cells C. As above outlined, the substrate 12 may comprise a tissue portion forming part of a live being (human, animal or plant) or may comprise a tissue or a culture of cells (animal or plant) separated from a live being or a culture of micro-organisms (bacteria or fungi, e.g. yeast).

Substrate 12 is also applied with a substance (organic or inorganic or biopolymeric) 30 to be introduced into the cells C. The substance 30 may be applied in a number of different ways, some of which are listed below by way of non-limiting examples:

. direct application of the substance to the substrate 12, e.g. by applying to the substrate a fluid containing the substance;

. indirect application of the substance, e.g. by introducing the substance into the circulatory system of the tissue portion forming the substrate; and

.injecting the substance, e.g. using needlelike

electrodes 6,7 (not shown), each having an inner conduit containing the substance to be injected into the tissue portion forming the substrate. The substance may also be injected using needles separate from the electrodes.

The substance 30 introduced may be inorganic or organic or biopolymeric, e.g.

- a nucleic acid;
- a DNA molecule containing regulatory sequences and sequence coding for therapeutic genes or genes of interest for biomedical or biotechnological purposes;
- an oligonucleotide, whether natural (phosphodiesters) or modified (inside the backbone of the oligonucleotide, such as phosphosulfates, or at the extremities, by addition of groups to protect the oligonucleotides from digestion of nucleases; the description of oligonucleotide modifications being non-limiting);
- a protein or peptide, whether natural or genetically or chemically modified, extracted from natural sources or obtained by synthesis, or a molecule simulating the structure of a protein or peptide, whatever its structure;
- a cytotoxic agent, in particular, the antibiotic bleomycin or the cisplatinum;

- a penicillin; and
- other pharmacological agents.

The substrate 12 can also be treated without the application of a substance when the purpose is to extract from the cells C a molecule (organic or inorganic or biopolymeric) contained in or produced by the cells C. In particular, the production of proteins or small organic molecules produced by genetically modified cells or genetically selected cells could be collected from the producing cells by the controlled procedure achieved by the device here described. Extraction of substances from cells C can be achieved by diffusion through the permeabilized membranes, by reverse iontophoresis or by any other active, passive or combined mechanism.

Electroporation device 1 is activated to generate one pulse or a train of pulses (block 100) that are spaced one with respect the other. Electroporation of the cells is therefore started and the ratio **GT** begins to fall following the first portion of curve $\mathbf{C_{GT}}$ **(i.e.** $\mathbf{C_{GT}}$**-I)**.

Pulses are applied for a period of time (block 110) so that ratio **GT** reaches its minimum at the time **Tm**; for instance period **Td** may be 15 μs.

Then to avoid damages in the cells an immediate check is performed (blocks 120 and 130) to see if, after

the minimum has been reached, curve C_{GT} has a too rapid increase (**dG** > 1); in fact, a too rapid increase after the minimum is a clear indication of irreversible damages to the cells (to that regard see curve $C_{GT\text{-}IRREVERSIBLE}$ shown in figure 3). In case of a detected indication of irreversible damages a corrective action is performed (blocks 140 and 145) by immediately decreasing the voltage applied thus preventing final damage to the cells. The check of blocks 120, 130 may be substituted (or integrated) by the check of blocks 126 and 127.

In case that no indication of damage is detected then the average slope of curve C_{GT} is scrutinised (block 150) to see if and how the cells are being permealized. In particular:

- if the calculated average variation **ΔG** of ratio **GT** falls within the reference interval ($0 < \Delta G < \Delta G_{obb}$) a situation of normal beginning of the process of permeabilization of the cells is detected and the process of permeabilization is normally continued by increasing the voltage (block 170) so that the value of **ΔG** tends to the expected value ΔG_{obb};
- if the calculated average variation **ΔG** of ratio **GT** falls outside the reference interval and it is smaller than both limits delimiting the

interval ($\Delta G < 0 < \Delta G_{obb}$) no beginning of permeabilization is detected (with this regard see curve $C_{GT\text{-}NO\text{-}PERM}$) and the voltage is increased (block 180) to start the process of permeabilization; and

- if the calculated average variation ΔG of ratio **GT** falls outside the reference interval and it is greater than both limits delimiting the interval ($\Delta G > \Delta G_{obb} > 0$) a potentially dangerous situation is detected and a corrective action is consequently performed (blocks 140 and 145).

The above operation may also be performed by using, instead of the ratio **GT** $= i_e/v_p$, any mathematical combination of current i_e and voltage v_p.

Moreover, the above operations may also be performed by using, instead of any mathematical combination of current i_e and voltage v_p, the value of the current i_e. In fact, current i_e has a shape that is very similar to the shape of ratio **GT** presented in figure 3 if Vp rise times is very fast and if Vp is then constant or almost constant.

In the above case all the operations disclosed with respect to blocks 100-200 directly performed on the current **ie**. For instance block 120 determines the instantaneous variation **die** of current **ie**, block 130 compares the instantaneous variation **die** with reference

values, block 150 calculates an average variation **Δie** of current **ie** and block 160 compares the calculated average variation **Δie** with reference values.

According to the above embodiment, it is necessary a more simple equipment for measure and calculation and the electroporation device provide a stimulating signal extremely "square".

It is therefore clear that, according to the present invention, the curve C_{GT} is constantly monitored and the stimulating signal is applied in a modified manner according to the detected waveform of an initial portion of curve C_{GT}.

In particular, the application of the stimulating signal is dependent on the shape of the curve C_{GT}, in particular the slope at particular time points.

Studies and experiments performed by the applicant have revealed that controlling the process of permeabilization as above outlined, i.e. focusing the analysis in the initial part of the waveform that corresponds to the initial instants wherein the process has been started but the real permeabilization has not still occurred, permits from one side to avoid damages to the cells and from the other side to obtain a good permeabilization of the cells.